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First detection of '*Candidatus* Phytoplasma ulmi' in Switzerland and in *Orientus ishidae* Matsumura, 1902

Alan Oggier[1], Christophe Debonneville[2], Marco Conedera[1], Olivier Schumpp[2], Attilio Rizzoli[3]

1 *Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Campus di Ricerca, A Ramél 18, 6593, Cadenazzo, Switzerland*

2 *Agroscope, Route de Duillier 60, 1260, Nyon, Switzerland*

3 *Agroscope, Campus di Ricerca, A Ramél 18, 6593, Cadenazzo, Switzerland*

https://zoobank.org/44820AB3-6D26-43D4-91D1-3F5B2143E69A

Corresponding author: Attilio Rizzoli (attilio.rizzoli@agroscope.admin.ch)

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Abstract

'*Candidatus* Phytoplasma ulmi' (*Ca.* P. ulmi) belongs to the ribosomal subgroup 16SrV-A and is associated with dieback, shoot proliferation and yellows disease on various *Ulmus* spp. Other plant species, such as *Carpinus betulus* and *Prunus* spp. have also been reported infected by the same pathogen. In 2021, in the frame of research activities focused on grapevine's Flavescence dorée (FD), one specimen of *Orientus ishidae* - an East Palearctic leafhopper that was identified as an alternative vector of FD phytoplasmas - was found harboring *Ca.* P. ulmi in southern Switzerland. No phytoplasmas were detected in plant samples taken in the same location. *Orientus ishidae* has already been reported to be able to acquire diverse phytoplasmas associated with other plant diseases, such as Peach X-disease. This is the first report of *Ca.* P. ulmi in Switzerland, as well as in *O. ishidae*. *Ca.* P. ulmi may potentially be present in the wild compartment of the Swiss Pre-alpine and Alpine range, but no dedicated survey has so far been conducted. In the case of *O. ishidae*, this finding highlights the broad affinity of such a species for the acquisition of several phytoplasmas. This calls for a further investigation regarding its potential role as a vector on various pathosystems of agronomic importance.

Key Words

alien species, leafhopper, Neobiota, phytoplasma, vector

Introduction

Phytoplasmas are cell wall-less bacteria associated with the degradation of plant tissues and the manipulation of metabolic activity often leading to important agronomic losses (Bertaccini 2022). The infection of healthy plants is caused by the feeding activity of insect vectors, which passively acquire the pathogen from infected plant specimens. Once acquired, phytoplasmas multiply inside the insect's organs. The infected vector becomes potentially infectious once the phytoplasmas reach the salivary glands (Bertaccini et al. 2019).

Among the diseases associated with phytoplasmas, grapevine's Flavescence dorée (FD) is one of the most insidious and destructive ones, causing important economic losses to European viticulture (Tramontini et al. 2020). The phytoplasmas associated with FD and provisionally classified as '*Candidatus* Phytoplasma vitis' (FDp) belong to the ribosomal subgroups 16SrV-C and D (Firrao et al. 2004; Lee et al. 2004). Considering the high risk of spread and the detrimental losses linked to FD, FDp is a quarantine organism in the European Union and Switzerland (Fedlex 2018; Tramontini et al. 2020). The epidemic spread of FD is caused by the Nearctic leafhopper *Scaphoideus titanus* Ball, 1932 (Hemiptera, Cicadellidae, Deltocephalinae) (Schvester et al. 1961; Chuche and Thiéry 2014). However, alternative host plant species, as well as alternative and putative vectors were identified. Among these, the leafhopper *Orientus ishidae* Matsumura, 1902 (Hemiptera, Cicadellidae, Deltocephalinae) was identified as an alternative vectoring agent of FDp in trials conducted by Lessio et al. (2016).

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Moreover, and most importantly for the particular case of southern Switzerland, several studies showed that *O. ishidae* may locally play a role in the maintenance of FD, mostly in the landscape (Casati et al. 2017; Jermini et al. 2019; Malembic-Maher et al. 2020; Rizzoli et al. 2021). *Orientus ishidae* is native to Asia and was found in 1998 for the first time in Europe (Guglielmino 2005), where it now spread to most of the continent (EPPO Global Database 2023). Some of the host plants of *O. ishidae* are *Corylus avellana*, *Acer* spp., *Alnus glutinosa*, *Salix* spp. and *Carpinus betulus* (Nickel 2010; Rizzoli et al. 2021), which are very common in European forests and thus, quite often found near vineyards. Some of these species, such as *A. glutinosa* and *C. avellana* have already been reported harboring FDp genotypes (Arnaud et al. 2007; Casati et al. 2017; Malembic-Maher et al. 2020; Rizzoli et al. 2021; Kogej Zwitter et al. 2023) and may thus be involved in alternative epidemiological cycles of FDp. Moreover, *O. ishidae* is able to oviposit on grapevines and gone-wild grapevines, thus exacerbating the risk of FDp flow between the cultivated and the wild compartment when newly hatched nymphs have a direct access to infected plant material (Lessio et al. 2019; Oggier et al. 2023). In addition to FDp, *O. ishidae* is able to acquire '*Candidatus* Phytoplasma pruni' belonging to subgroup 16SrIII-A and associated with Peach X-disease (Rosenberg and Jones 1978). Recently, Dalmaso et al. (2023) also reported the capability of *O. ishidae* to acquire '*Candidatus* Phytoplasma mali' associated with Apple Proliferation (16SrX-A) in field trials conducted in Trentino-Alto Adige (Italy), the most important region for apple production in Italy and the Alpine region.

This manuscript reports the first finding of '*Candidatus* Phytoplasma ulmi' (*Ca.* P. ulmi, ribosomal subgroup 16SrV-A) in Switzerland and in *O. ishidae*. *Ca.* P. ulmi is associated with dieback, shoot proliferation and yellows disease on various *Ulmus* spp. (Lee et al. 2004). So far, it has been recorded in several European countries, such as Italy (Pisi et al. 1981), Germany (Mäurer et al. 1993), and France (Boudon-Padieu et al. 2004). In addition to *Ulmus* spp., *Ca.* P. ulmi may infect other plant species, as recently observed by Rigamonti et al. (2023), who found infected hosts belonging to the species *Carpinus betulus*, *Prunus domestica*, and *P. ulmifolius* in Northwestern Italy. To date, *Scaphoideus luteolus* Van Duzee, 1894 (Hemiptera, Cicadellidae, Deltocephalinae), *Macropsis glandacea* Fieber, 1868 (Hemiptera, Cicadellidae, Eurymelinae, syn. *Macropsis mendax*), and *Amplicephalus curtulus* Linnavuori & DeLong, 1977 (Hemiptera, Cicadellidae, Deltocephalinae) have been identified as competent vectors of *Ca.* P. ulmi in the United States of America, Italy, and Chile, respectively (Baker 1949; Carraro et al. 2004; Arismendi et al. 2014). To our knowledge, no previous studies were ever conducted on the Palearctic species *M. glandacea* in relation to phytoplasmas in Switzerland. As for *S. luteolus* and *A. curtulus*, no further indication regarding their potential presence in continental Europe is known.

Materials and methods

Study area and experimental design

The study area comprised Canton Ticino (southern slope of the Swiss Alps). In the frame of current research activities regarding the FD epidemics in vineyards and their surroundings, experimental plots are monitored for the presence of FDp vectors and grapevines carrying symptoms linked to Grapevine Yellows are routinely marked and sampled for molecular analysis. Leafhopper populations in vineyards are surveyed with a minimum amount of six yellow sticky traps (YST; Rebell Giallo, Andermatt Biocontrol AG, Switzerland) hanged on the highest wire of the training system. In the surrounding landscape, at least four YST mounted on wooden sticks (ca. 1.50 m off the ground) are placed in the direct proximity of spontaneous plant species, such as *A. glutinosa*, *C. avellana*, *Acer* spp., etc., which are known to host alternative FDp vectors (e.g., *O. ishidae*). In 2021, 16 new plots were added to the standard design to monitor the possible presence of FDp vectors in the surroundings of gone-wild grapevines and/or rootstock resprouts originating from incorrect or incomplete rogueing of former vineyards located in the direct proximity of currently cultivated vineyards (Oggier et al. 2023). In these additional plots, YST were generally placed in fewer amounts and later during the season (from August onwards). The following year, the monitoring design was enhanced by adding additional YST and by extending the sampling period, which started in mid-July, in order to increase the chances of capturing FDp vectors imagoes during the population peak. The landscape surrounding each plot was described using parameters, such as dominant tree species, including presence and abundance of known host plant species of either FDp and/or alternative vectors, such as *O. ishidae* (e.g., *A. glutinosa*, *C. avellana*, *Salix* spp., etc.). If available, a set of random leaves originating from rootstocks and gone-wild grapevines was collected during the month of September in 2021 and 2022.

Insect and leaf processing

Leafhoppers determination was conducted using a stereo microscope (Olympus SZX16 with SDF PLAPO 1XPF objective lenses, made in Japan). For the particular case of *O. ishidae*, the morphological key provided by Günthart and Mühlethaler (2002) was used. After detaching target insects from YST using Glurex forte (D-Limonene 50–100%; Andermatt Biocontrol AG, Switzerland) and Ethanol (70% v/v), the specimens were transferred into tubes with Ethanol (99% v/v) and stored at −20 °C. Grapevine leaves were excised with scissors. The petioles and the major veins were then frozen at −20 °C until further processing.

Nucleic acid extraction and phytoplasma detection

Each insect was individually homogenized in 900 µL of extraction buffer (3% Cetyltrimethylammonium bromide CTAB, 1.4 M NaCl, 25 mM EDTA, 1 M Tris-HCl, 2 µL β-Mercaptoethanol, pH 8.0) and shaken for 30 min at 600 rpm and 65 °C. 900 µL of Chloroform/Isoamyl-alcohol was added, homogenized by vortexing for 5 s and centrifuged for 5 min at 3000× *g*. The aqueous layer was carefully transferred to a new tube, mixed with an equal volume of cold Isopropanol, and incubated 60 min at −20 °C for DNA precipitation. Precipitated material was recovered by 2 min of centrifugation at 10000× *g* and washed with 1 mL of 70% Ethanol. A DNA pellet was dried overnight at room temperature and resuspended into 100 µL of PCR-grade water. For plant samples, 0.5 to 1 g of petioles and midribs from 3 to 4 different leaves per specimen were ground in 6 mL of extraction buffer using a Homex grinder (Bioreba). Subsequently, 2 mL of this homogenate was centrifuged for 10 min at 1000× *g*. 900 µL of the supernatant was processed as described above.

The presence of 16SrV group phytoplasma in the samples was assessed by quantitative PCR analysis according to Hodgetts et al. (2009). Cycling conditions were 5 min at 95 °C followed by 42 cycles of 15 sec at 95 °C and 1 min at 60 °C, using a CFX96 real-time PCR instrument (Bio-Rad). PCR amplifications were carried out in 25 µL reactions using 20 pmol of forward and reverse primer, 1 to 2 µL of DNA template, with GoTaq G2 Flexi DNA polymerase (Promega) following manufacturer's instructions. The *map* and *imp* genes loci were amplified by nested PCR according to Arnaud et al. (2007) and Trivellone et al. (2019), respectively.

Sequencing and data analysis

PCR products were controlled by electrophoresis on a 1% agarose gel and purified by ultrafiltration with NucleoFast 96 PCR plates (Macherey-Nagel). Products were sent to Fasteris (Plan-les-Ouates, Switzerland) for forward and reverse sequencing using Sanger technology. Trees were inferred by maximum likelihood method in MEGA using the General Time Reversible model and bootstrapping with 500 replicates. All trees were visualized with iTOL (https://itol.embl.de, accessed on 14 September 2023; Letunic and Bork 2021).

Results

In the frame of the research activities associated with FD, we analyzed 16 *O. ishidae* specimens caught in 2021 (out of 26 YST) and 267 caught in 2022 (out of 85 YST) for a total of 283 insects. One of the three specimens of *O. ishidae* caught in 2021 in the additional plot of Cugnasco (WGS84 coordinates 46.18037, 8.91938) was found harboring *Ca.* P. ulmi. The infected insect was captured between 1 and 14 October 2021. All specimens caught the following year in the same plot resulted free of *Ca.* P. ulmi ($N = 6$, $N_{YST} = 1$). No other tested insect species, such as *S. titanus* was found infected by *Ca.* P. ulmi (data not shown). The four grapevines sampled in the plot of Cugnasco in 2021 resulted free of external symptoms linked to Grapevine Yellows and phytoplasma.

The sequence of the *secY-map* gene obtained from the infected *O. ishidae* is 100.00% identical to the reference strain AM384900 *Ca.* P. ulmi, isolate E04-D438 found in France, Loire Atlantique, previously reported by Arnaud et al. (2007). The comparison of the *imp* gene also confirmed the detection of *Ca.* P. ulmi (isolates MT668492, MT668497, MT668435). The amplicon has 1 single nucleotide polymorphism (99.78% identity) compared to the sequence of MT668492 (*Ca.* P. ulmi isolate 4319 Ug from Germany). The sequence was deposited on the NCBI database under accession number OR594266. The phylogenetic trees in Fig. 1 show the relationship between the sequences of the *secY-map* and the *imp* genes, respectively, obtained in this work and reference strains (see Table 1 for GenBank Accession no. and further details).

Discussion

This communication reports the first account of *Ca.* P. ulmi in Switzerland, as well as the first record of *O. ishidae* harboring such phytoplasmas, in general. *Ca.* P. ulmi was identified during routine molecular analyses conducted in the frame of an ongoing research project focused on FDp in vineyards and in the adjacent wild compartment in the Swiss southern Alps.

'*Candidatus* Phytoplasma ulmi' may already be present in Swiss *Ulmus* spp., as well as in other host plant species both on the Alpine range and on the Swiss Plateau. However, no proper surveys have so far been conducted in order to confirm its presence and potential impact on mountainous and Alpine ecosystems. Most interestingly, *O. ishidae* appears to be able to acquire other phytoplasma strains in addition to genotypes belonging to the ribosomal subgroups 16SrV-C and 16SrV-D (Mehle et al. 2010; Casati et al. 2017; Rizzoli et al. 2021), 16SrIII-A (Rosenberg and Jones 1978), and 16SrX-A (Dalmaso et al. 2023). However, in the specific case of *Ca.* P. ulmi in the study area, the infection rate for *O. ishidae* seems to be very low (1/283), when considering the molecular analyses conducted in 2021 and 2022. The substantial difference of captured specimens between 2021 and 2022 was mostly due to the period of sampling, which in 2021 was shorter and did not cover the population's peak. *Orientus ishidae* is present in almost all continental Europe including mountainous Pre-alpine and Alpine ranges (EPPO Global Database 2023). Host plant species of particular agronomic importance, such as grapevine and apple (De Meyer 2014; International Organisation of Vine and Wine 2023), are widely cultivated in the same contexts and spontaneous woody plant species, such as *Ulmus* spp.

Table 1. Phytoplasma isolate from *Orientus ishidae* obtained in this work and reference strains along with the relative GenBank accession number, compared genes, host, geographic origin and reference.

Sample ID or reference strain	GenBank accession no.	Gene(s)	Host	Phytoplasma strain and genotype	Geographic origin	Reference
Ca. P. ulmi isolate Cugnasco CH	OR594266	*map imp*	*Orientus ishidae*	*Ca.* P. ulmi	Switzerland, Canton Ticino	This work
Ca. P. ulmi isolate E04-D438	AM384900	*secY map*	*Ulmus minor*	*Ca.* P. ulmi	France, Loire Atlantique	Arnaud et al. (2007)
Ca. P. ulmi isolate EY18_SRB	HM038478	*secY map*	*Ulmus laevis*	*Ca.* P. ulmi	Serbia	Jović et al. (2011)
Ca. P. ulmi isolate NK16	KU202150	*secY map*	*Ulmus laevis*	*Ca.* P. ulmi	Croatia	Katanić et al. (2016)
Ca. P. ulmi isolate E04-D714	AM384901	*secY map*	*Ulmus glabra*	*Ca.* P. ulmi	France, Haute-Vienne	Arnaud et al. (2007)
Ca. P. ulmi isolate EY1	GU004330	*map cds*	Plant leaf material	*Ca.* P. ulmi	USA	Lee et al. (2010)
map M50 isolate VF-06-27-5	LT221945	*map*	*Vitis vinifera*	*Ca.* P. vitis FD1 M50	France, Acquitaine	Malembic-Maher et al. (2020)
map M38 isolate AI-031-08	LT221933	*map*	*Alnus glutinosa*	*Ca.* P. vitis FD2 M38	Italy, Veneto	Malembic-Maher et al. (2020)
map M51 isolate VS-Loza37	LT221946	*map*	*Vitis vinifera*	*Ca.* P. vitis FD3 M51	Serbia	Malembic-Maher et al. (2020)
map M54 isolate VF06-30-18	LT221949	*map*	*Vitis vinifera*	*Ca.* P. vitis FD2 M54	France, Acquitaine	Malembic-Maher et al. (2020)
Ca. P. ziziphi isolate Jwb-nky	CP025121	*map*	*Ziziphus jujuba* Mill	*Ca.* P. ziziphi	China	Wang et al. (2018)
4319_Ug_SN	MT668492	*imp*	*Ulmus glabra*	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
5554_Ug_MV	MT668497	*imp*	*Ulmus glabra*	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
0865_Ug_BYs	MT668435	*imp*	*Ulmus glabra*	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
2261_Ug_HE	MT668459	*imp*	*Ulmus glabra*	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
2226_Ug_HE	MT668458	*imp*	*Ulmus glabra*	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
4167_Ul_SN	MT668491	*imp*	*Ulmus* sp.	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
ULW	MT418908	*imp*	*Ulmus minor*	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
2732_Um_BB	MT668465	*imp*	*Ulmus* sp.	*Ca.* P. ulmi	Germany	Schneider et al. (2020)
FD70	MT668500	*imp*	*Vicia faba*	*Ca.* P. vitis FD70	France	unpublished
ALY1	MT668499	*imp*	*Alder* sp.	Alder yellows phytoplasma	Germany	Schneider et al. (2020)
FD-D	MK614707	*imp*	*Vitis vinifera*	*Ca.* P. vitis FD-D	Italy	Trivellone et al. (2019)
Ca. P. ziziphi isolate Jwb-nky	MG818479	*imp*	Chinese jujube	*Ca.* P. ziziphi	China	unpublished

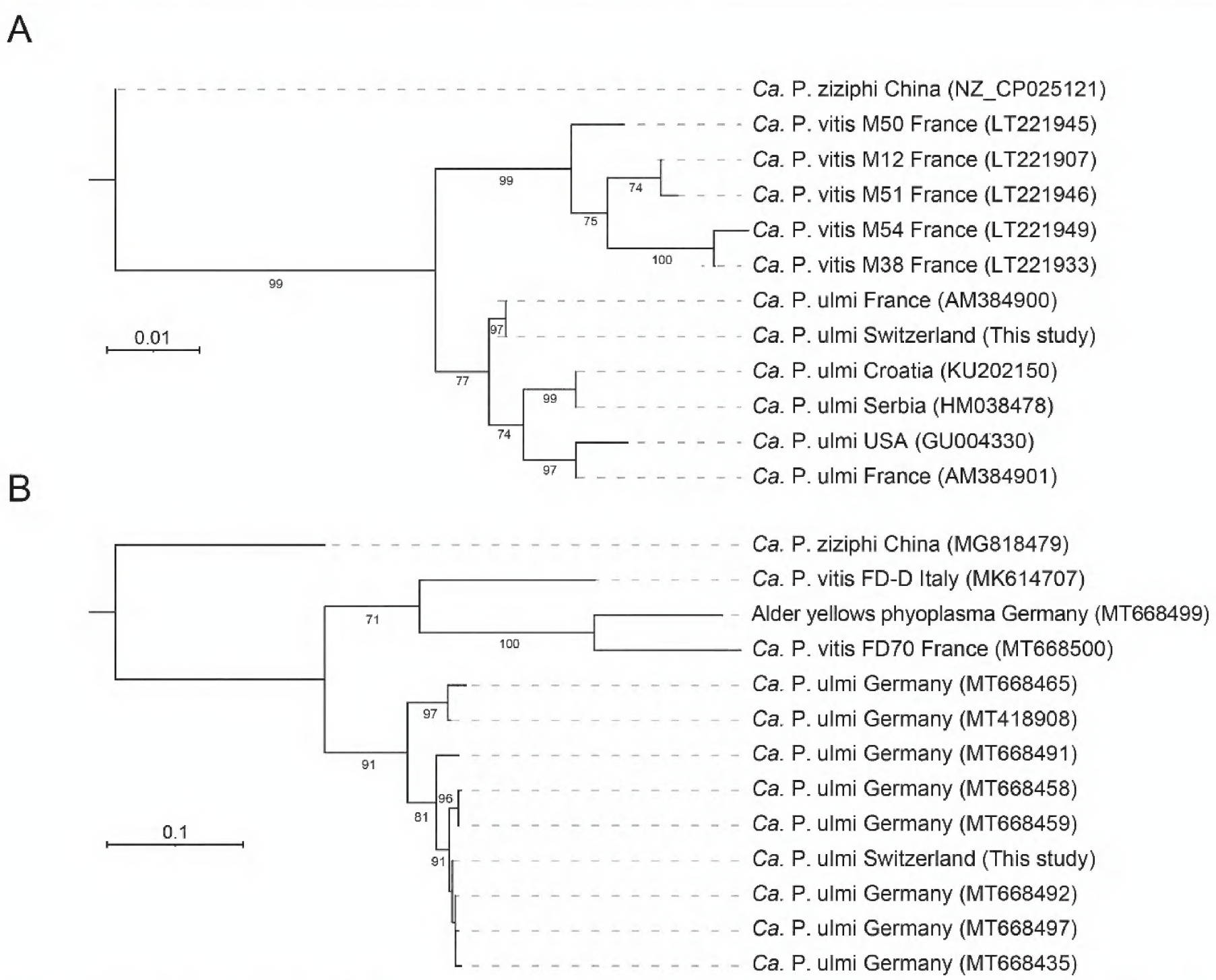

Figure 1. Phylogenetic tree of the *secY-map* (**A**) and *imp* (**B**) genes sequences from *Orientus ishidae* obtained in this work and reference strains from Genbank (see Table 1). Maximum likelihood phylogeny based on nucleotide sequences of (**A**) *map* (543 bp) and (**B**) *imp* (465 bp) genes. The numbers on branches indicate the level of bootstrap support (500 replicates). Support values above 70% are labeled. The scale bar shows the number of substitutions per site.

and *C. avellana* are very common in the whole geographic range. Therefore, *O. ishidae* seems to be a wild-card in several pathosystems and further research is needed in order to better understand its ecology and potential impact on agriculture, as well as on the overall health of spontaneous woody plant species inhabiting different ecosystems, including the whole Alpine range.

Author contributions

Alan Oggier: Conceptualization, investigation, formal analysis, data curation, writing - review and editing. **Christophe Debonneville:** Methodology, investigation, formal analysis, visualization, software, validation, writing - review and editing. **Marco Conedera:** Writing – review and editing, funding acquisition, project administration. **Olivier Schumpp:** Writing – review and editing, resources, validation. **Attilio Rizzoli:** Conceptualization, methodology, writing – original draft, writing – review and editing, validation, supervision.

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